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14. ABSTRACT
We have identified NuSAP as prognostic marker upregulated in recurrent prostate tumors. Our grant aimed at identifying the role of NuSAP in promoting proliferation and invasion in Prostate Cancer and identify genes that upregulate NuSAP expression. To characterize the role and regulation of NuSAP in prostate cancer, we studied the expression of NuSAP in the LNCaP and PC3 human prostate cancer cell lines. Post-transcriptional silencing of the NuSAP gene severely hampered the ability of PC3 to invade and proliferate *in vitro*. The promoter region of the NuSAP gene contains 2 CCAAT boxes and binding sites for E2F. Transient transfection of an E2F1 cDNA and 431 bp of the NuSAP promoter demonstrated E2F1 as an important regulator of expression. Deletion of the E2F binding site at nt -246 negated the effects of E2F1 on NuSAP expression. Electrophoretic mobility shift assays demonstrated that nuclear extracts of cells over-expressing E2F1 bound directly to the E2F binding site in the NuSAP promoter region. Finally, immunohistochemistry showed a strong correlation between E2F1 and NuSAP expression in human prostate cancer samples. NuSAP is a novel biomarker for prostate cancer recurrence after surgery and its over-expression appears to be driven in part by E2F1 activation.

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Introduction:
We have identified NuSAP as prognostic marker upregulated in recurrent prostate tumors. Our grant aimed at identifying the role of NuSAP in promoting proliferation and invasion in Prostate Cancer and identify genes that upregulate NuSAP expression. Nucleolar and spindle-associated protein (NuSAP) is an essential microtubule- and chromatin-binding protein found in the proliferating cells. Its primary function is to induce extensive bundling and stabilization of spindle microtubules against depolymerization and cross-link large numbers of microtubules into aster-like structures and thick fiber networks during metaphase. Interestingly, both excessive amount and knockdown of NuSAP leads to disruption of cell division. Thus, NuSAP must be tightly controlled during cell cycle progression. However, how NuSAP protein is controlled and the precise role of NuSAP in regulation of cell cycle still remains unclear.

Specific Aim 1: profile the expression of NuSAP, C-Myc, RanGTP, NF-YA, c/EBPα and AR in Prostate Cancer Cell Lines. Investigate invasion and proliferation:
In the initial application we proposed to investigate the transcript level of NuSAP, c-MYC, NFYA, CEBPA and AR in prostate cancer cell lines. Based on the transcript profiles we found *NuSAP* expressed at relatively high levels in the prostate cancer cell lines LNCaP and PC3 (data not shown). These two cell lines hence became our model to investigate the role of NuSAP in prostate cancer. As stated in the initial application, knockdown of *NuSAP* transcript levels significantly decreased proliferation of PC3 cells *in vitro* compared with control cells (Figure 1a). In addition, knockdown of *NuSAP* transcript levels significantly decreased invasion to <5% compared with controls in which 40% of the cells invaded through the membrane (Figure 1b). Similarly, knockdown of *NuSAP* in LNCaP cells significantly decreased proliferation (not shown). However, because wild-type LNCaP cells were poorly invasive, we could not assess the effects of *NuSAP* knockdown on invasion in this cell line.

KEY RESEARCH ACCOMPLISHMENTS:

- Investigated the transcript levels of NuSAP, MYC, NFYA and AR in prostate cell lines.
- Demonstrated that upon knock down of NuSAP there is a significant decrease in the growth and invasion profile of in both PC3 and LNCaP cell lines.

Specific Aim 2: To understand the mechanisms of action of NuSAP by testing its effects on androgen receptor signaling and gene expression

So far, using the 431bp NuSAP promoter we have not been able to identify a binding site for Androgen Receptor (AR). We have expanded our search to look at the larger part of the promoter region to look for AR binding regions or elements that could have a direct link to AR. There has been an AR enhancer region identified 100kb upstream of NuSAP transcription start site (Waltering et al., 2009). This enhancer region has been demonstrated to influence NuSAP transcript levels under varying concentration of Androgen. At present we are actively looking into designing experiments to experimentally validating this region and its influence on NuSAP gene expression. Additionally, we are also looking at the Rb and MYC pathways since both have binding sites within 1000bp of NuSAP promoter region and also been experimentally shown to influence AR expression (Sharma et al., 2010).

KEY RESEARCH ACCOMPLISHMENTS:

- Aanalyzed extended NuSAP promoter region to identify an AR enhancer region which could have an influence on NuSAP expression.

Specific Aim 3: To define the important regulatory elements that influence NuSAP gene expression levels in prostate cancer cells.

To understand the underlying mechanisms of NuSAP over-expression in aggressive prostate cancers, we investigated the promoter sequences of the NuSAP gene. Previously, NFYA and MYC have been implicated as transcriptional regulators of NuSAP (Fujiwara et al., 2006). Using MATCH™ software (TRANSFAC), we investigated whether there might be other potential transcription factor binding sites in the 5'-upstream region of the NuSAP gene. As expected, 2 putative NFYA (-139/-144 and -310/-315) binding sites were identified, although no MYC binding sites were observed. Interestingly, one E2F (-246/ -252) binding site also was identified (Figure 2a). We cloned 431bp of the human NuSAP promoter region and created 5 deletion mutants containing the putative NFYA and E2F regulatory elements. The cloned NuSAP promoter constructs were ligated into pGL4.11-luc luciferase vector and the promoter-reporter constructs were verified by direct sequencing. LNCaP and PC3 prostate cancer cell lines were transiently transfected with 431bp promoter region and the 5 deletion constructs and luciferase activity was assayed. In both PC3 and LNCaP cell lines, constructs containing -123 to -431 of the NuSAP promoter showed comparable luciferase activity which essentially decreased to background levels in the constructs lacking those sequences, suggesting this region harbors critical regulatory elements (Figure 2b and 2c).

To further investigate the regulatory elements within the -431 to -123 region, we co-transfected the 431bp NuSAP promoter-luciferase reporter in tandem with cMyc or NFYA cDNAs into LNCaP and PC3 cell lines. As anticipated, MYC and NFYA resulted in 6- and 4-fold respectively higher luciferase activity above baseline activity (Figure 2d and 2e). Increased expression of the E2F1 transcription factor has previously been suggested to be prognostic for prostate cancer (Davis et al., 2006; Malhotra et al., 2011), but has not been implicated as a regulator of NuSAP gene expression. When E2F1 and the NuSAP promoter-reporter construct were co-transfected into LNCaP and PC3 cell lines, luciferase activity increased 3-fold above baseline (Figure 3a and 3b). Targeted mutation of the E2F binding sequence (TTTGGCGC to TTTGATAC) ablated E2F1 enhanced expression.

To demonstrate whether E2F1 directly interacts with NuSAP gene promoter sequences, we prepared nuclear extracts from E2F1 transfected LNCaP and PC3 cell lines and ran a mobility shift assay using a 24 bp biotin labeled oligonucleotide probe corresponding to the –237 to -261 of NuSAP promoter region containing the E2F binding site (Figure 4). Nuclear extracts from both LNCaP and PC-3 bound the NuSAP E2F promoter sequences suggesting a direct interaction between E2F1 and the NuSAP promoter. We were able to compete away binding with unlabeled probe and eliminate binding using an anti-E2F1 antibody (BD- Pharmingen, USA).

The further test whether E2F1 and NuSAP show coordinated expression *in vivo*, we performed immunohistochemical staining with NuSAP and E2F1 antibodies of a tissue microarray containing 121 primary prostate cancers (Figure 5a and 5b). Each core was evaluated for nuclear over-expression of E2F1 and NuSAP. An individual core was regarded as "positive" if the neoplastic cells showed unambiguous (2+ or greater) nuclear staining intensity on a 0-3 scale. The number of neoplastic cells showing positive nuclear reactivity was also counted in each core

for each antibody. 35 cancer samples showed focal nuclear immunoreactivity for both proteins, 20 cancers were positive for E2F1 alone, 10 were positive for NuSAP alone and 47 were negative for both proteins (X^2 = 20.8, p= 0.000) (Figure 5c). Since Gleason grade is a powerful predictor of prostate cancer aggressiveness, we also looked at the association between Gleason grading of the TMA core and the staining profile of both antibodies. Tumors with higher Gleason grades showed significant increased in numbers of cells positive for E2F1 (P < 0.000). Interestingly, the number of cells positive for NuSAP staining did not correlate with Gleason grade (*P*=0.8) (Figure 5d).

KEY RESEARCH ACCOMPLISHMENTS:

- Amplified at 431bp human NuSAP promoter from a DNA sample.
- Validated potential NFYA and E2F binding sites within 431bp of NuSAP promoter.
- Demonstrated that E2F could directly bind to NuSAP promoter and influence the expression of NuSAP.

REPORTABLE OUTCOMES:

A research publication titled **"Increased expression of *NuSAP* in recurrent prostate cancer is mediated by *E2F1*"** (attached) was published in a Nature publication, Oncogene in Febuary 2012.

Overall:

We have made significant progress on all 3 aims. In the second year of funding, we have planned to investigate the biological influence of Rb/E2F1 and cMYC on NuSAP. Both Rb/E2F1 and MYC have been found commonly perturbed in the prostate cancer especially in aggressive prostate cancers. Investigation into both these pathways will also expand our understanding into role of AR as both E2F1 and MYC have been demonstrated to directly influence the expression of AR.

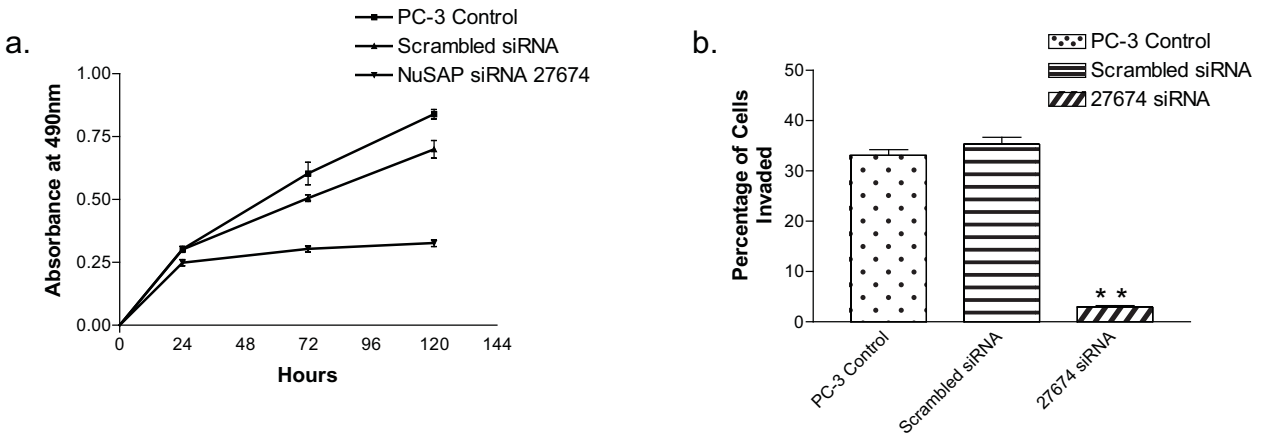

Figure 1. NuSAP knockdown reduces proliferation and invasion in PC3 cells. a) Cells transfected with NuSAP siRNA;Silencer Select 27674 show significantly decreased cell viability/proliferation, measured by the MTS assay. b) Cells transfected with NuSAP siRNA;Silencer Select 27674 display significantly decreased cell invasion through Matrigel. ***P*<0.001 compared to scrambled vector.

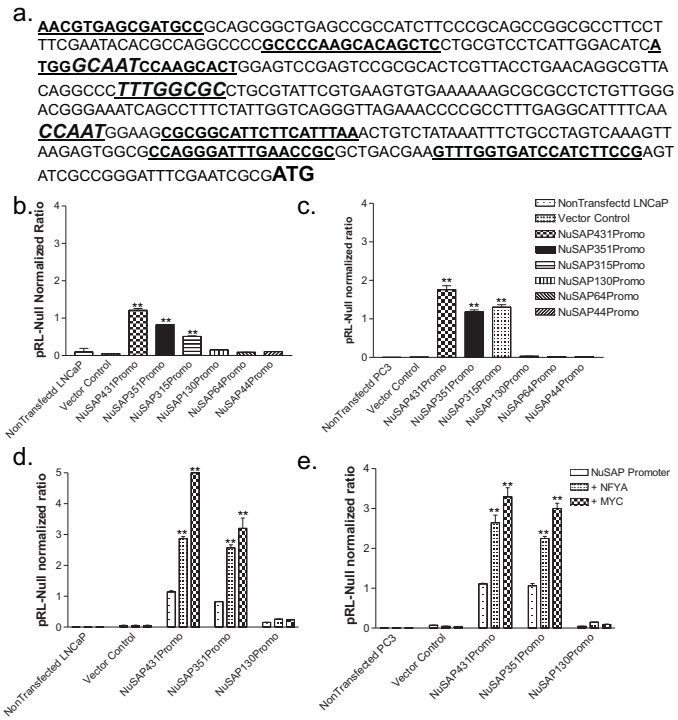

Figure 2. Promoter analysis of the human NuSAP gene. a) The 431bp human NuSAP promoter sequence. **Bold underlined:** primer sequences for amplifying NuSAP promoter deletion constructs. ***Bold Italics:*** Transcription factor binding sequences for NFYA (GCAAT and CCAAT) and E2F (TTTGGCGC). b) Baseline expression levels of the 431bp human NuSAP gene promoter and 5 deletion constructs ligated to pGL4.11-luciferase in LNCaP cells. The bp -123 to -431 region of the NuSAP promoter induces expression of NuSAP in LNCaP cells. c) Baseline expression for the promoter constructs in PC3 cells shows identical regulation. d) Co-transfection of LNCaP cells with NFYA and MYC increases NuSAP expression above baseline in LNCaP cells in the bp -123 to -431 region. e) Similar regulation by MYC and NFYA in PC3 cells. Data represents relative luciferase activity (firefly luciferase activity versus renilla luciferase activity) which was calculated in each cell line and are the mean values from three separate experiments. ** P < 0.001 compared to vector controls.

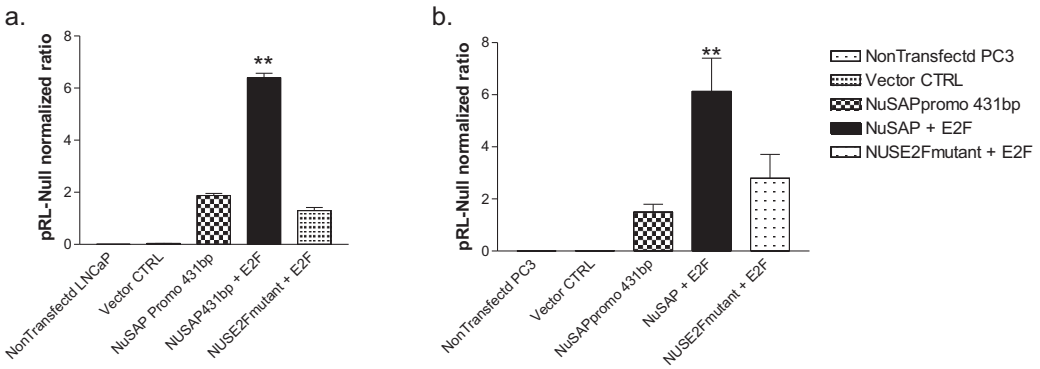

Figure 3. E2F1 enhances NuSAP expression. Co-transfection of 431bp NuSAP promoter and E2F1 results in significantly increased NuSAP reporter expression in a) LNCaP and b) PC3 cell lines. Mutation of E2F binding sequence in NuSAP promoter negates the effects of E2F1. Data represents relative luciferase activity (firefly luciferase activity versus Renilla luciferase activity) which was calculated in each cell line. Data are the mean values from three separate experiments. ** P <0.001 compared to cells not transfected with E2F1.

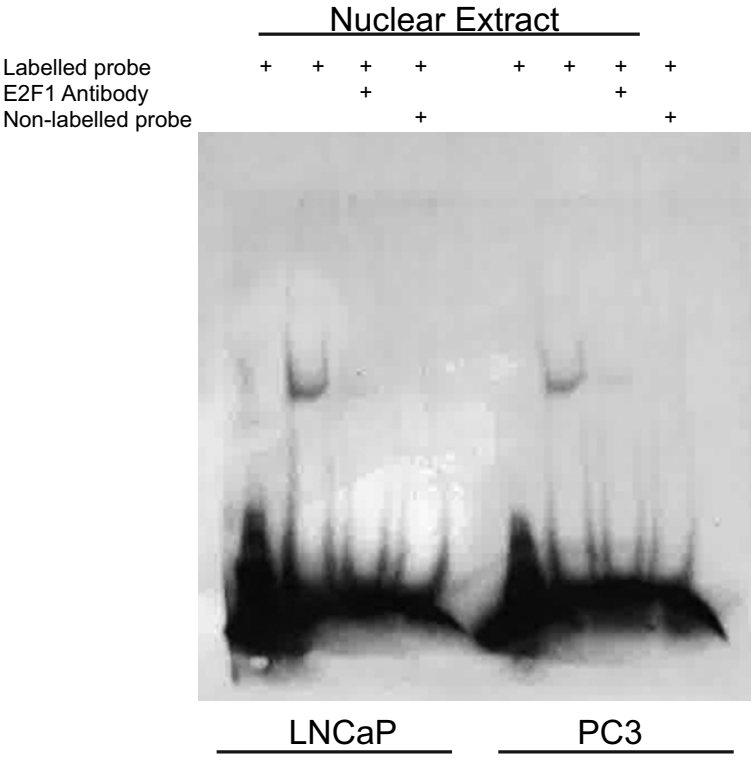

Figure 4. E2F1 directly binds to NuSAP promoter. Nuclear extracts from both LNCaP and PC3 cell lines were incubated with a biotin labeled oligonucleotide probe corresponding to the E2F binding site in the NuSAP promoter at bp -246/-254 (lanes 2, 6). Binding could be competed away by co-incubation with an anti-E2F1 antibody (lanes 3, 7) and with 100-fold molar excess of unlabeled probe (lanes 4, 8).

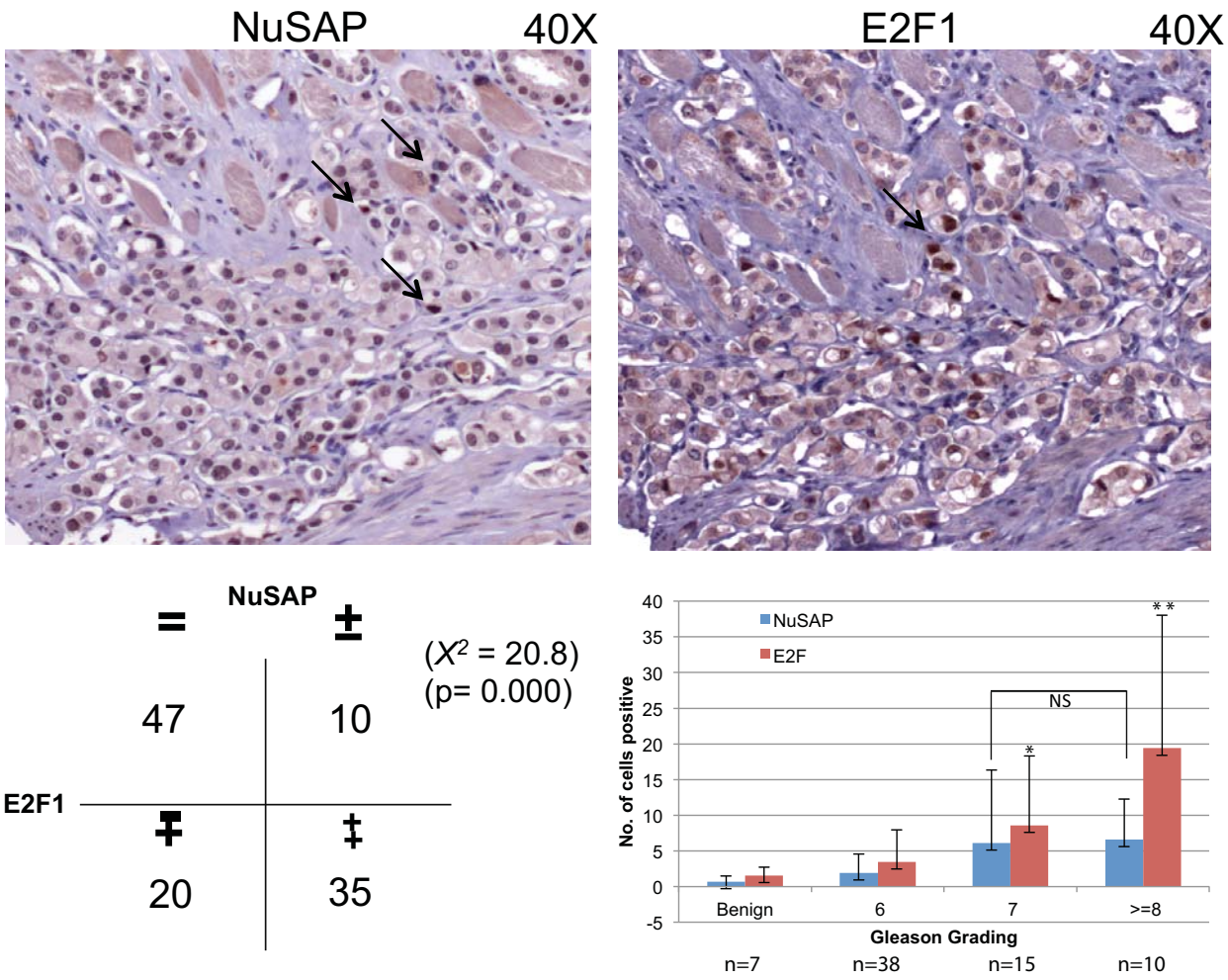

Figure 5. Immunostaining of prostate TMA with NuSAP and E2F1. a) Nuclear staining of NuSAP observed in isolated prostate cancer nuclei (arrows). b) Nuclear staining of E2F1 in an adjacent section of prostate cancer. c) Correlation between NuSAP and E2F1 staining in 121 prostate cancer specimens on a tissue microarray. d) Number of nuclei with positive staining per 1 mm core of prostate cancer tissue on the tissue microarray separated by Gleason grade of the core. * $P < 0.05$, ** $P < 0.001$ compared to benign tissue.

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ORIGINAL ARTICLE

Increased expression of *NuSAP* in recurrent prostate cancer is mediated by *E2F1*

ZG Gulzar[1], JK McKenney[2] and JD Brooks[1]

Increasing evidence suggests that prostate cancer is overdiagnosed and overtreated, and prognostic biomarkers would aid in treatment selection. To define prognostic biomarkers for aggressive prostate cancer, we carried out gene-expression profiling of 98 prostate tumors and 52 benign adjacent prostate tissue samples with detailed clinical annotation. We identified 28 transcripts significantly associated with recurrence after radical prostatectomy including *NuSAP*, a protein that binds DNA to the mitotic spindle. Elevated *NuSAP* transcript levels were associated with poor outcome in two independent prostate cancer gene-expression datasets. To characterize the role and regulation of *NuSAP* in prostate cancer, we studied the expression of *NuSAP* in the LNCaP and PC3 human prostate cancer cell lines. Posttranscriptional silencing of the *NuSAP* gene severely hampered the ability of PC3 to invade and proliferate *in vitro*. The promoter region of the *NuSAP* gene contains two CCAAT boxes and binding sites for E2F. Transient transfection of an *E2F1* cDNA and 431 bp of the *NuSAP* promoter demonstrated *E2F1* as an important regulator of expression. Deletion of the E2F-binding site at nucleotide −246 negated the effects of *E2F1* on *NuSAP* expression. Electrophoretic mobility shift assays demonstrated that nuclear extracts of cells overexpressing *E2F1* bound directly to the E2F-binding site in the *NuSAP* promoter region. Finally, immunohistochemistry showed a strong correlation between *E2F1* and *NuSAP* expression in human prostate cancer samples. *NuSAP* is a novel biomarker for prostate cancer recurrence after surgery and its overexpression appears to be driven in part by *E2F1* activation.

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Keywords: prostate cancer; gene-expression profiling; *NuSAP*; *E2F1*; recurrence

INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer in the western world. An estimated 648 400 men will be diagnosed with prostate cancer this year and 136 500 will die of their disease.[1] The disparity between the number of men with prostate cancer and the number of men who die of their disease is largely due to the wide variation in the behavior of prostate cancer. Only a fraction of patients have tumors capable of metastasizing and causing death. One of the most important challenges in treating prostate cancer is in identifying patients with potentially lethal tumors needing aggressive treatment and those with indolent tumors that can be safely watched. The European Randomized Study of Screening for Prostate Cancer trial estimated that the number of prostate-specific antigen (PSA)-detected prostate cancers treated to save one man's life was 48.[2] A recent analysis that was restricted to the men who were actually screened suggested a smaller ratio of treated cancers at 30 to save one man's life at 10 years.[3]

Many investigators have documented significant molecular diversity in human prostate cancer and this heterogeneity undoubtedly contributes to the spectrum of clinical behavior. Several groups have shown that comprehensive gene-expression profiling can capture molecular features that distinguish normal from cancerous prostate, and a few have identified genes associated with prognosis.[2,4–11] However, many of these studies have been small in size and on samples with minimal clinical annotation, limiting their ability to identify prognostic markers or provide insights into the biology of prostate cancer. To identify gene-expression signatures associated with recurrence after definitive prostate cancer surgery, we performed gene-expression profiling of 98 primary prostate tumor samples from 86 patients with detailed clinical annotation and clinical follow-up. From this analysis, we identified overexpression of the nucleolar and spindle-associated protein (*NuSAP*) gene as an important marker of prognosis in prostate cancer. Functional studies confirm the potential biological importance of the *NuSAP* gene in prostate cancer and identify *NuSAP* as a potential end-target of *E2F1*.

RESULTS

We performed gene-expression profiling on 98 tumors from 86 individuals using HEEBO (human exonic evidence-based oligonucleotide) spotted microarrays containing 44 544 70-mer probes. The prostate tissue samples were harvested from men who underwent radical retropubic prostatectomy for clinically localized prostate cancer at Stanford University by a single surgeon (JDB) between 1998 and 2007. Detailed clinical data, including follow-up and recurrence status, were available in 90 patients (92%). Mean patient age, preoperative serum PSA levels, clinical stage and pathological Gleason grade were compatible with the risk profiles of contemporary patients undergoing surgery for prostate cancer (Supplementary Table S1).

Screen-detected prostate cancers found in contemporary surgical series are rarely lethal even after a decade of follow-up. Recurrence after surgery has been associated with a more aggressive clinical course and lethality, and has therefore been used as a surrogate endpoint of prostate cancer aggressiveness in many studies.[12] To identify genes that are associated with

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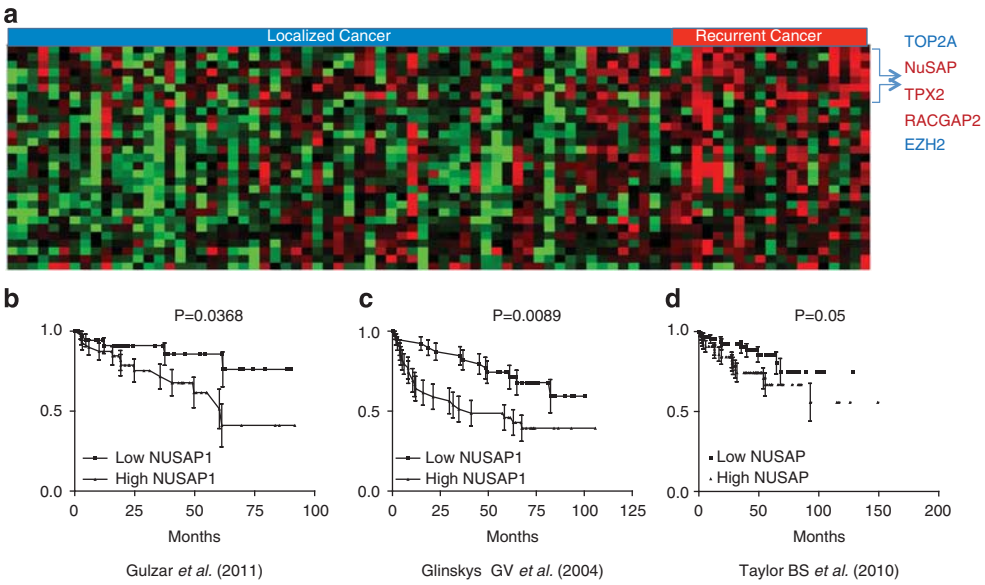

Figure 1. (**a**) Two-class SAM survival analysis comparing 19 recurrent and 63 nonrecurrent prostate cancer samples. A false discovery rate of 4% resulted in 28 gene transcripts differentially expressed between the two groups. Each tumor sample is represented in a column and individual transcripts are displayed in rows. Red indicates relative increased expression level of transcripts relative to the median level across the samples, whereas green represents relative decrease in expression levels, and the degree of color saturation corresponds to the degree of change. (**b**) Kaplan–Meier survival analysis of *NuSAP* gene expression performed in our dataset. Tumor samples were divided into two groups based on whether the *NuSAP* gene expression value was above or below the median value. (**c**) Increased expression of *NuSAP* is prognostic in that dataset from Glinsky GV *et al.*[34] and (**d**) Taylor BS *et al.*[35] prostate datasets. *P*-values calculated using the log-rank test.

biochemical recurrence following radical prostatectomy, we performed a statistical analysis of microarray (SAM) survival analysis using all 83 of the tumor samples that had associated clinical follow-up. SAM survival analysis uses Cox modeling to identify genes whose expression levels are significantly associated with time to biochemical recurrence. Because enrichment for highly variable genes improves the performance of SAM, we selected 1600 genes that varied by at least five-fold across the entire dataset and used these to perform SAM survival analysis. From this, 28 transcripts were identified that were significantly associated with recurrence after radical prostatectomy at FDR of 4% (Figure 1 and Supplementary Table S2). Among the 28 genes, there were several (*KIAA0101*, *TOP2*, *EZH2*, *IGFBP3*, *RAC2*, *RCSD1*, *CYP2D6*, *MCM6*, *Versican*, *HGF* and *ETV5*) that have been reported to be prognostic in a variety of malignancies including prostate cancer.[13–32] More than half of the transcripts have not been previously implicated as prognostic and included *NuSAP*, *TPX2*, *RACGAP1*, *CP*, *SIPA1L2*, *CF1*, *LOC391426*, *EST_AA496936*, *THBS2*, *SCUBE2*, *CERK*, *CRABP2*, *ENO1*, *AK2*, *CYP2D6*, *LOC285296*, *BCR/TCR_IGKV1/OR-2* and *APOC1*.

Interestingly, *TPX2*, *RACGAP1* and *NuSAP* are important members of the microtubule and mitotic spindle regulation pathway and were found upregulated in recurrent samples compared with nonrecurrent samples. Both *NuSAP* and *TPX2* are indispensable proteins required for microtubule stabilization and cross-linking in response to local generation of RanGTP and its regulator *RACGAP1*. *NuSAP* has been associated with poor prognosis in human melanoma[33] but has never been linked to outcomes in other solid tumors. Expression levels of *NuSAP* in the prostate samples were validated by qPCR on 20 (4 adjacent normal, 8 nonrecurrent and 8 recurrent) prostate samples and excellent concordance was found with the microarray data (Supplementary Figure S1).

We hypothesized that *NuSAP* might have an important role in prostate cancer progression and aggressiveness. Not surprisingly, when cancers in our dataset were segregated into groups with *NuSAP*-expression levels above and below the median value, tumors with higher *NuSAP* levels had a significantly increased risk of biochemical recurrence after surgery ($P<0.01$ by log-rank test; Figure 1b). To validate this observation, we investigated the relationship between *NuSAP*-expression levels and outcomes in two independent prostate cancer microarray datasets. Both of these datasets have used a large cohort of patients with well-characterized prostate tumor samples and associated clinical follow-up.[34,35] Increased *NuSAP* expression levels were significantly associated with recurrence after radical prostatectomy ($P<0.01$ and $P=0.05$, log rank test; Figures 1c and d).

NuSAP is expressed at relatively high levels in the prostate cancer cell lines LNCaP and PC3. To evaluate the possible effects of *NuSAP* overexpression in prostate cancer we knocked down expression levels of *NuSAP* in the prostate cancer cell line PC3. Cells were transiently transfected with either the *NuSAP* siRNA; Silencer Select 27674 (Invitrogen–Life Technologies, Grand Island, NY, USA) or the scrambled siRNA control, and *NuSAP* transcript levels were evaluated by qPCR (Figure 2b and Supplementary Figure S2). Knockdown of *NuSAP* transcript levels significantly decreased proliferation of PC3 cells *in vitro* compared with control cells (Figure 2a). In addition, knockdown of *NuSAP* transcript levels significantly decreased invasion to $<5\%$ compared with controls in which 40% of the cells invaded through the membrane (Figure 2b). Similarly, knockdown of *NuSAP* in LNCaP cells significantly decreased proliferation (not shown). However, because wild-type LNCaP cells were poorly invasive, we could not assess the effects of *NuSAP* knockdown on invasion in this cell line.

To understand the underlying mechanisms of *NuSAP* overexpression in aggressive prostate cancers, we investigated the promoter sequences of the *NuSAP* gene. Previously, *NFYA* and *MYC* have been implicated as transcriptional regulators of *NuSAP*.[36,37] Using MATCH Software-Biobase Biological Databases (Beverly, MA, USA) (TRANSFAC), we investigated whether there might be other potential transcription factor-binding sites in the 5′-upstream region of the *NuSAP* gene. As expected, two putative *NFYA*-binding sites ($-139/-144$ and $-310/-315$) were identified, although no *MYC*-binding sites were observed. Interestingly, one

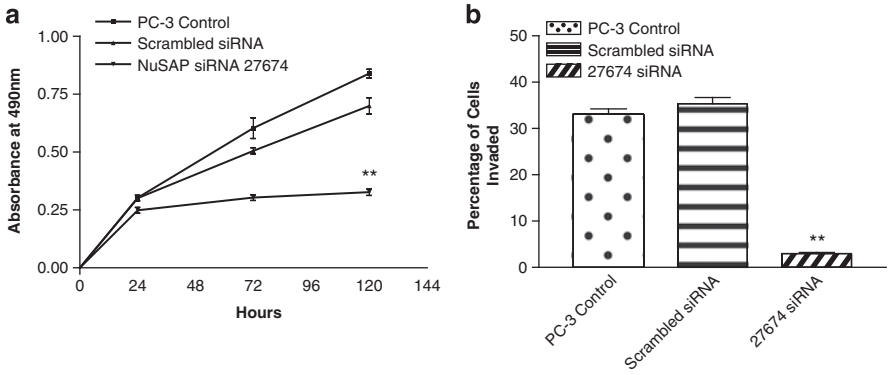

Figure 2. *NuSAP* knockdown reduces proliferation and invasion in PC3 cells. (**a**) Cells transfected with *NuSAP* siRNA;Silencer Select 27674 show significantly decreased cell viability/proliferation, measured by the MTS assay. (**b**) Cells transfected with *NuSAP* siRNA;Silencer Select 27674 display significantly decreased cell invasion through Matrigel. $^{**}P<0.001$ compared with scrambled vector.

E2F-binding site (−246/−252) also was identified (Figure 3a). We cloned 431 bp of the human *NuSAP* promoter region and created five deletion mutants containing the putative *NFYA* and E2F regulatory elements. The cloned *NuSAP* promoter constructs were ligated into pGL4.11- luciferase vector and the promoter-reporter constructs were verified by direct sequencing. LNCaP and PC3 prostate cancer cell lines were transiently transfected with 431 bp promoter region and the five deletion constructs, and luciferase activity was assayed. In both PC3 and LNCaP cell lines, constructs containing −123 to −431 of the *NuSAP* promoter showed comparable luciferase activity, which essentially decreased to background levels in the constructs lacking those sequences, suggesting this region harbors critical regulatory elements (Figures 3b and c).

To further investigate the regulatory elements within the −431 to −123 region, we co-transfected the 431-bp *NuSAP* promoter luciferase reporter in tandem with cMyc or *NFYA* cDNAs into LNCaP and PC3 cell lines. As anticipated, *MYC* and *NFYA* resulted in six- and four-fold, respectively, higher luciferase activity above baseline activity (Figures 3d and e).

Increased expression of the *E2F1* transcription factor has previously been suggested to be prognostic for prostate cancer[38,39] but has not been implicated as a regulator of *NuSAP* gene expression. When *E2F1* and the *NuSAP* promoter-reporter construct were co-transfected into LNCaP and PC3 cell lines, luciferase activity increased three-fold above baseline (Figures 4a and b). Targeted mutation of the E2F-binding sequence (5′-TTT GGCGC-3′ to 5′-TTTGATAC-3′) ablated *E2F1*-enhanced expression.

To demonstrate whether *E2F1* directly interacts with *NuSAP* gene promoter sequences, we prepared nuclear extracts from *E2F1*-transfected LNCaP and PC3 cell lines and ran a mobility shift assay using a 24-bp biotin-labeled oligonucleotide probe corresponding to the −237 to −261 of *NuSAP* promoter region containing the E2F-binding site (Figure 5). Nuclear extracts from both LNCaP and PC-3 bound the *NuSAP* E2F promoter sequences, suggesting a direct interaction between *E2F1* and the *NuSAP* promoter. We were able to compete away binding with unlabeled probe and eliminate binding using an anti-*E2F1* antibody (BD-Pharmingen, San Diego, CA, USA).

To further test whether *E2F1* and *NuSAP* show coordinated expression *in vivo*, we performed immunohistochemical staining with *NuSAP* and *E2F1* antibodies of a tissue microarray containing 121 primary prostate cancers (Figures 6a and b). Each core was evaluated for nuclear overexpression of *E2F1* and *NuSAP*. An individual core was regarded as 'positive' if the neoplastic cells showed unambiguous (2+ or greater) nuclear staining intensity on a 0–3 scale. The number of neoplastic cells showing positive nuclear reactivity was also counted in each core for each antibody. In all, 35 cancer samples showed focal nuclear immunoreactivity for both proteins, 20 cancers were positive for *E2F1* alone, 10 were positive for *NuSAP* alone and 47 were negative for both proteins ($X^2=20.8$, $P=0.000$) (Figure 6c). Because Gleason grade is a powerful predictor of prostate cancer aggressiveness, we also looked at the association between Gleason grading of the TMA core and the staining profile of both antibodies. Tumors with higher Gleason grades showed a significant increase in numbers of cells positive for *E2F1* ($P<0.000$). Interestingly, the number of cells positive for *NuSAP* staining did not correlate with Gleason grade ($P=0.8$) (Figure 6d).

DISCUSSION

Through gene-expression profiling, we identify *NuSAP* and a candidate biomarker for recurrence after radical prostatectomy. Overexpression of *NuSAP* is associated with recurrence of prostate cancer and this finding was validated in two independent datasets. It is particularly notable that out of the 28 transcripts associated with biochemical recurrence after surgery, 3 of them (*NuSAP*, *TPX2* and *RACGAP1*) are members of the microtubule-associated protein family that regulate mitotic spindle organization. *NuSAP* appears to be critical for mitotic spindle assembly and for binding of DNA to the microtubules. Our data suggest that *NuSAP* has an important functional role in some aggressive prostate cancers.

One obvious explanation for the role of *NuSAP* in prostate cancer is that it is a marker for proliferation. Increased expression of proliferation biomarkers has been shown to be prognostic in many cancer types.[40-42] A recent analysis of gene sets shown to be prognostic in breast cancer suggests that they all reflect increased proliferation in aggressive cancers.[40] Several studies have demonstrated that biomarkers of increased proliferation connote poor prognosis in prostate cancer and that measurement of several markers of prognosis simultaneously improves outcome prediction.[39] Therefore, *NuSAP* represents a promising candidate biomarker to add to an immunohistochemical panel of prognostic biomarkers.

Because *NuSAP* is correlated with more aggressive prostate cancers, we sought to understand the mechanisms by which it becomes overexpressed in some prostate cancers. *NuSAP* is located on chromosome 15q15.1, a region not implicated in copy number alterations or other structural alterations in human prostate cancers.[5,43] Whole-genome sequencing of several prostate cancers has not identified mutations within or near the *NuSAP* gene.[44] We therefore investigated the 5′-regulatory elements of the *NuSAP* gene. Copy number gains of chromosome 8q in the region of the Myc gene are relatively common in prostate cancer. We confirmed that overexpression of Myc in prostate cells *in vitro* is associated with increased expression of a *NuSAP* promoter construct, despite the absence of canonical Myc-binding

a

AACGTGAGCGATGCCGCAGCGGCTGAGCCGCCATCTTCCCGCAGCCGGCGCCTTCCT
TTCGAATACACGCCAGGCCCC**GCCCCAAGCACAGCTC**CTGCGTCCTCATTGGACATC**A**
TGG*GCAAT*CCAAGCACTGGAGTCCGAGTCCGCGCACTCGTTACCTGAACAGGCGTTA
CAGGCCC***TTTGGCGC***CTGCGTATTCGTGAAGTGTGAAAAAAGCGCGCCTCTGTTGGG
ACGGGAAATCAGCCTTTCTATTGGTCAGGGTTAGAAACCCCGCCTTTGAGGCATTTTCAA
CCAATGGAAG**CGCGGCATTCTTCATTTAA**ACTGTCTATAAATTTCTGCCTAGTCAAAGTT
AAGAGTGGCG**CCAGGGATTTGAACCGC**GCTGACGAA**GTTTGGTGATCCATCTTCCG**AGT
ATCGCCGGGATTTCGAATCGCG**ATG**

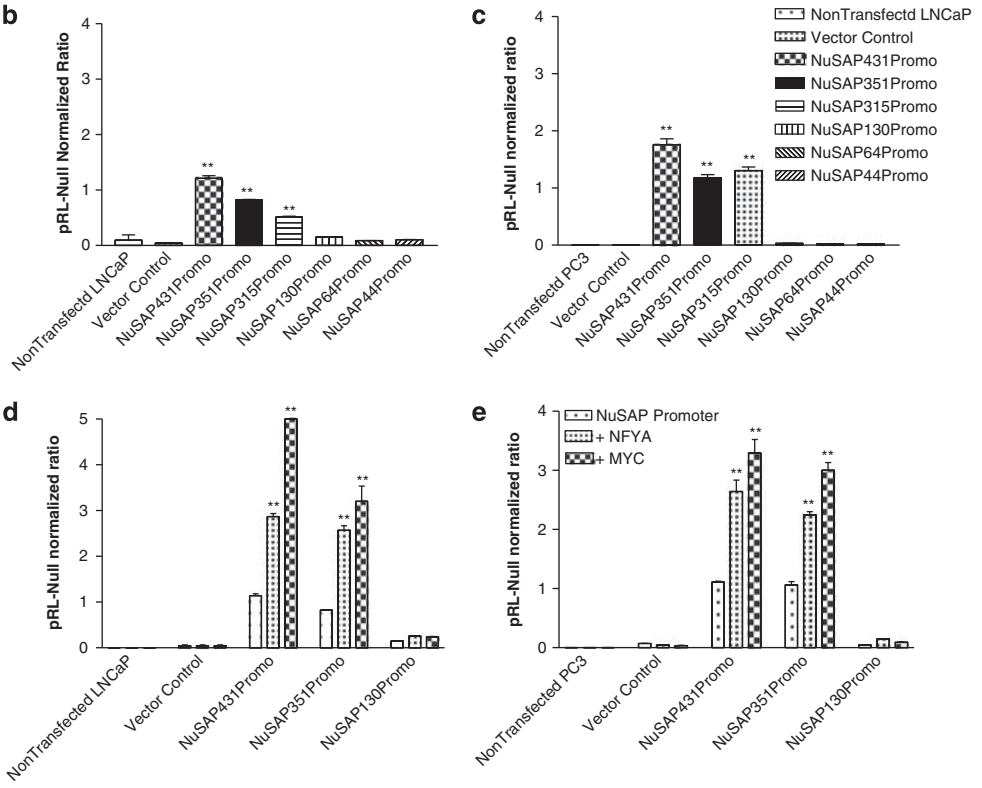

Figure 3. Promoter analysis of the human *NuSAP* gene. (**a**) The 431 bp human *NuSAP* promoter sequence. Bold underlines represent primer sequences for amplifying *NuSAP* promoter deletion constructs. Bold Italics represent transcription factor-binding sequences for *NFYA* (GCAAT and CCAAT) and E2F (TTTGGCGC). (**b**) Baseline expression levels of the 431-bp human *NuSAP* gene promoter and five deletion constructs ligated to pGL4.11–luciferase in LNCaP cells. The −123 to −431-bp region of the *NuSAP* promoter induces expression of *NuSAP* in LNCaP cells. (**c**) Baseline expression for the promoter constructs in PC3 cells shows identical regulation. (**d**) Co-transfection of LNCaP cells with NFYA and *MYC* increases *NuSAP* expression above baseline in LNCaP cells in the −123 to −431-bp region. (**e**) Similar regulation by *MYC* and *NFYA* in PC3 cells. Data represent relative luciferase activity (firefly luciferase activity versus Renilla luciferase activity) that was calculated in each cell line and are the mean values from three separate experiments. $^{**}P < 0.001$ compared with vector controls.

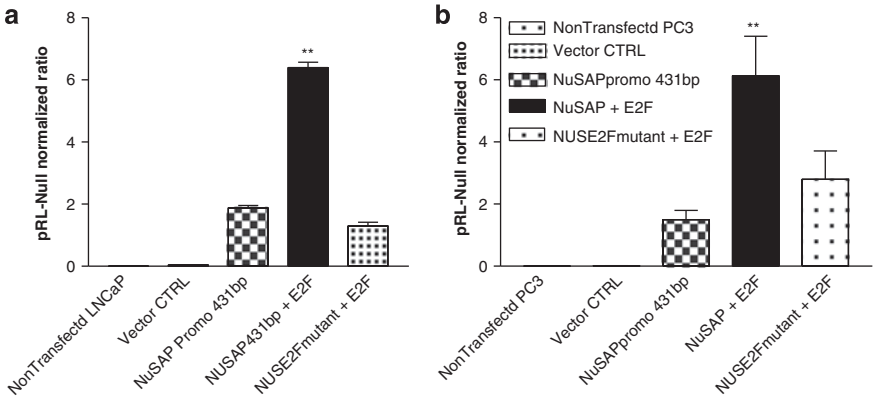

Figure 4. *E2F1* enhances *NuSAP* expression. Co-transfection of 431 bp *NuSAP* promoter and *E2F1* results in significantly increased *NuSAP* reporter expression in (**a**) LNCaP and (**b**) PC3 cell lines. Mutation of E2F-binding sequence in *NuSAP* promoter negates the effects of *E2F1*. Data represent relative luciferase activity (firefly luciferase activity versus Renilla luciferase activity) that was calculated in each cell line. Data are the mean values from three separate experiments. $^{**}P < 0.001$ compared with cells not transfected with *E2F1*.

sequences in this region. Although *NFYA* also increased expression of the *NuSAP* promoter-reporter construct, *NFYA* is an unlikely regulator of *NuSAP* in prostate cancer because it is expressed constitutively and *NuSAP* and *NFYA* transcript levels were not correlated in our dataset.[36] The *NuSAP* promoter does harbor an E2F-binding site and we were able to document that *E2F1* overexpression results in increased expression of a *NuSAP* promoter-reporter construct. In addition, deletion of this binding site abrogates expression, and *E2F1* physically interacts with the binding sequence in the *NuSAP* gene promoter. Because *E2F1* has been correlated with aggressive prostate cancer, it is a promising regulator of increased *NuSAP* expression in aggressive prostate cancers.

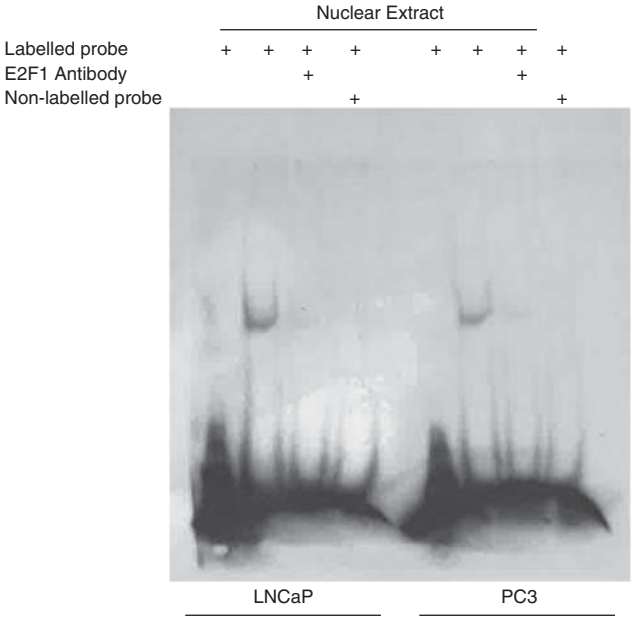

Figure 5. *E2F1* directly binds to *NuSAP* promoter. Nuclear extracts from both LNCaP and PC3 cell lines were incubated with a biotin-labeled oligonucleotide probe corresponding to the E2F-binding site in the *NuSAP* promoter at −246/−254 bp (lanes 2 and 6). Binding could be competed away by coincubation with an anti-*E2F1* antibody (lanes 3 and 7) and with 100-fold molar excess of unlabeled probe (lanes 4 and 8).

Identification of *E2F1* as a regulator of *NuSAP* has potential implications in understanding prostate cancer progression. E2F controls cell division by regulating the transcription of genes that are essential for DNA synthesis and cell cycle progression. Aberrant expression of *E2F1* has been documented in large number of malignancies including prostate cancer. Overexpression of *E2F1* has been found in invasive ductal breast carcinomas and non-small-cell lung carcinomas, where high levels of *E2F1* were associated with advanced disease and poor prognosis.[38] Increased expression of E2F transcript levels occurs as part of a proliferation cluster in a prostate cancer gene-expression dataset that we have reported previously. Expression of *E2F1* protein, along with proliferation markers Ki67 and TOP2A, is associated with an increased risk of recurrence after surgery in men with prostate cancer, independent of clinical stage, pretreatment serum PSA levels and tumor Gleason grade.[39] The retinoblastoma protein negatively regulates E2F and deletions of the Rb gene are relatively common events in prostate cancer. Recently, Sharma *et al*.[45] have suggested that the progression to castration-resistant prostate cancer is dependent on disruption of Rb that produces increased expression of the androgen receptor through the direct action of *E2F1*. Therefore, like androgen receptor, *NuSAP* could represent another important effector protein in the Rb-E2F regulatory pathway in prostate cancer. In addition, the activation and release of *NuSAP* from Imp α, Imp β and Imp 7 is mediated by

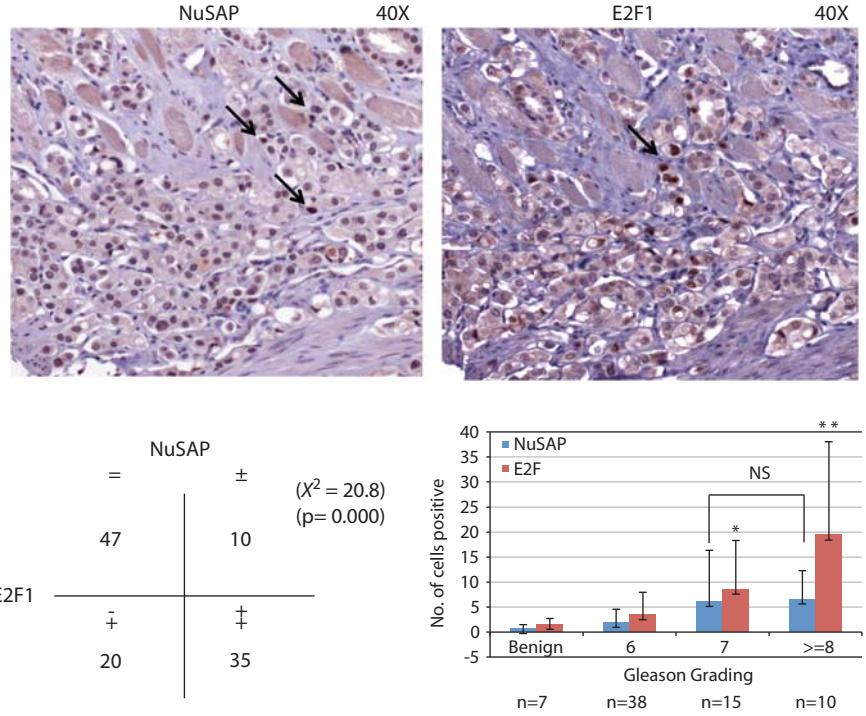

Figure 6. Immunostaining of prostate TMA with *NuSAP* and *E2F1*. (**a**) Nuclear staining of *NuSAP* observed in isolated prostate cancer nuclei (arrows). (**b**) Nuclear staining of *E2F1* in an adjacent section of prostate cancer. (**c**) Correlation between *NuSAP* and *E2F1* staining in 121 prostate cancer specimens on a tissue microarray. (**d**) Number of nuclei with positive staining per 1 mm core of prostate cancer tissue on the tissue microarray separated by Gleason grade of the core. *$P<0.05$, **$P<0.001$ compared with benign tissue.

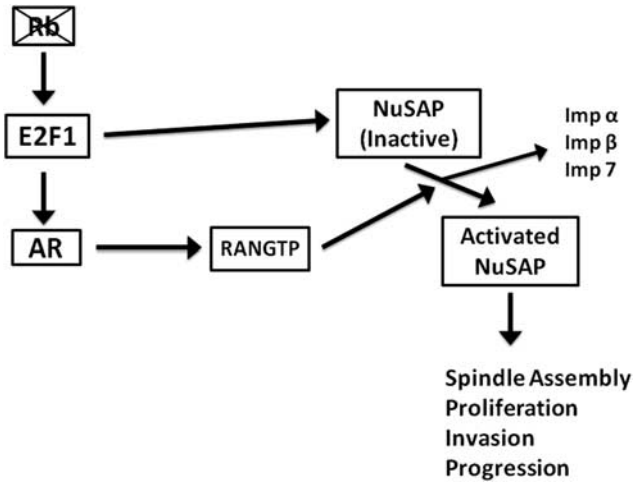

Figure 7. Schematic representation of possible role of *NuSAP* in the Rb-E2F signaling pathway including possible interactions with androgen receptor signaling.

RANGTP, an androgen receptor-regulated gene.[46] Therefore, *NuSAP* overexpression could cooperate to androgen receptor signaling in prostate cancer progression (Figure 7).

The functional consequences of *NuSAP* overexpression are somewhat unclear. *NuSAP* is indispensable to cell division and is selectively expressed in the proliferating cells. Its expression peaks during G2–mitosis phase and declines rapidly following cell division. *NuSAP* expression is highly correlated with cell proliferation during embryogenesis and adult life, and *NuSAP* deficiency in mice leads to early embryonic lethality.[47] In agreement with this finding, we found that knockdown of *NuSAP* in LNCaP and PC3 prostate cancer cell lines essentially stopped cell growth and significantly inhibited invasion of matrigel. *NuSAP* overexpression appears to be an end product of a regulatory pathway important in prostate cancer and might represent a critical effector protein in this pathway in its effects on the microtubules. Selective inhibition of *NuSAP*–mitotic spindle complex results in mitotic arrest, abnormal chromatin condensation, apoptosis and cell death. Based on its critical role in cell division, *NuSAP* could represent an important candidate target protein for therapy. For example, in acute myelogenous leukemia, some patients who undergo ablative chemotherapy and stem cell transplant subsequently develop autoantibodies against *NuSAP* and these antibodies are produced by the stem cell graft. Patients with autoantibodies against *NuSAP* show improved cancer remission rates and it is hypothesized that *NuSAP* is the direct target of a graft-versus-leukemia response.[44] Therefore, *NuSAP*, as an important effector protein in proliferation, could represent a novel therapeutic target in prostate cancer, melanoma and other malignancies.

MATERIALS AND METHODS

Sample collection

All prostate samples used for this study were collected at the Stanford University Medical Center between 1999 and 2007 with patient's informed consent under an Institutional Review Board (IRB)-approved protocol. Multiple tissue samples were harvested from each prostate, flash-frozen and stored at $-80\,^{\circ}C$. Frozen sections of each prostate sample were performed and evaluated by a genitourinary pathologist (JKM). The tumor and nontumor areas were marked and contaminating tissues were trimmed away from the block as described previously.[48] Tumor samples in which at least 90% of the epithelial cells were cancerous were selected for extraction of DNA and RNA. In total, we selected 98 tumors from 86 patients that met these criteria. Associated clinical data were collected and included preoperative PSA levels, clinical stage, pathological stage, tumor Gleason grade and clinical follow-up. Recurrence was defined as a measurable serum PSA (>0.1 ng/ml on two consecutive measurements) after surgery.

DNA/RNA extraction

The tumor and normal prostate tissue samples ($=<100$ mg) were homogenized for 1 min using P-2100 Polytron homogenizer (Polytron Homogenizer Capitol Scientific, Austin, TX, USA. The homogenates were centrifuged at 9000 r.p.m for 3 min and the supernatants were passed through a 21 g-needle four to five times before proceeding for DNA/RNA extraction. DNA, RNA and microRNA were isolated from each tissue sample using Qiagen AllPrep DNA/RNA mini kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol. RNA quality was assessed by the integrity of rRNA bands following gel electrophoresis.

Gene-expression profiling

Gene-expression profiling was performed as reported.[48] Briefly, Cy5-labeled cDNA was prepared by using 50 μg of total RNA from prostate samples and Cy3-labeled cDNA was prepared by using common reference mRNA (Stratagene-Agilent Technologies, Inc, Santa Clara, CA, USA), pooled from 11 established human cell lines. For each experimental sample, Cy5- and Cy3-labeled samples were cohybridized to HEEBO spotted microarrays.[49] HEEBO microarrays were manufactured in the Stanford Functional Genomics Facility at Stanford University and contained 44 544 70-mer probes. After hybridization, microarrays were imaged using an Axon GenePix 4000 scanner (Axon Instruments-Molecular Devices, LLC, Sunnyvale, CA, USA). Fluorescence ratios for array elements were extracted using GENEPIX software and uploaded into the Stanford Microarray Database for subsequent analysis.[50] Fluorescence ratios were normalized by mean centering genes for each array. Ratios were then mean-centered for each gene across all arrays within each of the four different array print runs used, to minimize potential print run-specific bias. Gene expression data have been deposited in GEO (ID no. pending).

Plasmid construction and mutagenesis

The human *NuSAP* promoter along with promoter deletion constructs were amplified from normal human genomic DNA and ligated to *Kpn*1/*Hin*dIII restriction site of the pGL4.11 luciferase reporter plasmid. Following primers were used to amplify the *NuSAP* promoter and its deletion constructs: (positions -1 to -431) Forward 5′-GGTACCCAACGTGAGC GATGCC-3′, Reverse 5′-AAGCTTCGCGATTCGAAATCCC-3′, (positions -1 to -352) Forward 5′-GGTACCGCCCCAAGCACAGCTC-3′, (positions -1 to -316) Forward 5′-GGTACCATGGGCAATCCAAGCACT-3′, (positions -1 to -130) Forward 5′-GGTACCCGCGGCATTCTTCATTTAA-3′, (positions -1 to -71) Forward 5′-GGTACCCCAGGGATTTGAACCGC-3′ and (position -1 to -45) Forward 5′-GGTACCGTTTGGTGATCCATCTTCCG-3′. The E2F-binding site within the *NuSAP* promoter was mutated using the QuikChange multi site-directed mutagenesis kit (Stratagene) based on the manufacturer's recommendations. The primers used for mutagenesis were Forward 5′-AGGCGTTACAGGCCCTTTGATACCTGCGTATTCGTGAAGTG-3′ and Reverse 3′-TCCGCAATGTCCGGGAAACTATGGACGCATAAGCACTTCAC-5′. Human *E2F1*, c-*MYC* and *NFYA*/NFDN (dominant negative) were kind gifts from Drs Julien Sage, Dean Felsher (Stanford University) and Roberto Mantovani (University of Milan).

Cell culture and transfection

LNCaP and PC-3 prostate cancer cell lines were grown in T-Medium and DMEM, respectively, supplemented with 10% fetal bovine serum. A total of 2×10^5 cells were transfected with 1.8 μg of firefly reporter plasmid (pGL4.11–Luc) containing the *NuSAP* gene promoter constructs and 200 ng of Renilla luciferase reporter plasmid (pRL-Null) using lipofectamine 2000 (Invitrogen) reagent according to the manufacturer's recommendations. After 48 h, cells were harvested and both firefly and Renilla luciferase activities in the cell extracts were determined by Dual Luciferase Assay kit (Promega, Madison, WI, USA). Co-Transfections of cells with either of *E2F1*,

NF-YA, NFDN, c-*MYC* and *NuSAP* promoter plasmids were done using the same protocol stated above keeping the ratio of Firefly to Renilla constant (1:0.1).

Cell proliferation and invasion assays

Cell proliferation was quantified using the MTS assay (Promega), a colorimetric assay based on detection of MTS tetrazolium compound (Owen's reagent) by metabolically active cells. Cell invasion was measured by a Boyden chamber assay (BD Biosciences, Franklin Lakes, NJ, USA). In all, 5000 PC3 cells per 24-well insert were seeded onto precoated filters (8 μm pore size, Matrigel 100 μg/cm^2), using a 0.5–10% fetal bovine serum gradient. After 24 h, cells traversing the filter were fixed with 10% buffered formalin, stained with crystal violet and manually counted. All the above assays were done in triplicate and all experiments were replicated at least once.

Nuclear extract and electrophoretic mobility shift assay

Nuclear extracts were prepared by using NE-PER Nuclear and Cytoplasmic Extraction reagents (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Nuclear extracts (10 μg) from *E2F1*-transfected LNCaP and PC3 cell lines were incubated for 15 min at room temperature with 20 nM of a biotin-labeled oligonucleotide probe containing a putative E2F-binding sequence from the *NuSAP* promoter in a 20 μl binding reaction containing 5 × binding buffer (final concentration 20 mM HEPES pH 8.0, 50 mM KCL, 1 mM DTT, 1 mM EDTA, 1 mM $MgCl_2$ and 5% glycerol) and 1 mg/ml Poly (dI-dC). The oligonucleotides sequences used were Forward 5′-CAGGCCC**TTTGGCGC**CTGCGTATT-3′ and Reverse 5′-GTCCGGG**AAACCG CG**GACGCATAA-3′. In order to demonstrate the specificity of the reaction, the extracts were also incubated with either of *E2F1* antibody (1 μg) (sc-22820, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or (2 μM) non-labeled probe for 15 min at room temperature prior to adding the labeled oligonucleotide. The probe-bound nuclear extracts were separated from the free probe in a 6% DNA retardation gel (Invitrogen) and the biotin-labeled probe was detected using the Phototope Star kit (New England Biolabs, Ipswich, MA, USA).

Immunohistochemistry

A standard two-layer strepavidin-biotin method was used to stain a prostate tissue microarray containing 121 cases. For *NuSAP* protein detection, sections were heated to 95 °C after adding Tris-EDTA solution (10 mM Tris–HCl, 1 mM EDTA, pH 9). *E2F1* antigen retrieval was carried out using 1 mM EDTA pH 9.0 and the samples were microwaved for 20 min. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol. Nonspecific binding was blocked with protein block serum-free reagent (Dako, Glostrup, Denmark). Sections were incubated overnight with rabbit anti-human *NuSAP* polyclonal antibody (Proteintech Group) (1:400) or mouse anti-*E2F1* monoclonal antibody (1:50; BD-Pharmingen, San Diego, CA, USA). Antibody binding was visualized with the Dako Envision System (Dako). The stained sections were reviewed and scored by a genitourinary pathologist (JKM). Only luminal epithelial cells displaying nuclear expression of *NuSAP* or *E2F1* were scored as positive.

Data analysis

For analysis of gene expression data, we included only well-measured genes whose expression varied, as determined[1] by signal intensity over background >1.5-fold in both test and reference channels in at least 75% of samples, [2] and 2-fold ratio variation from the mean in at least two samples. Transcripts associated with recurrence after surgery were identified using the SAM survival method.[51] Hierarchical clustering was performed and displayed using Cluster and TreeView software (Eisen Lab). Kaplan–Meier analysis and the log-rank test were performed using Prism Software version 2.01 (GraphPad Prism Software, Inc., La Jolla, CA, USA) to test the association between *NuSAP* expression levels and clinical outcome. The association between *NuSAP* and *E2F1* protein expression levels was carried out using Chi-square analysis. Comparison of the expression levels in the transfection assays was done by two-tailed Student's *t*-test using SPSS (IBM SPSS, Armonk, NY, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Author Contributions: ZGG, JDB and JKM agree with the manuscript's results and conclusions, analyzed the data, prepared tissue samples and contributed to the writing of the paper. ZGG and JDB designed the experiments/study. ZGG and JKM collected the data/did experiments for the study. ZGG wrote the first draft of the paper.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)